Resistance of Isolated Bacteria from Wastewater of Paper Factory in Basrah to Antibiotic and Mercuric Chloride

Eman A. Al-Imarah

University of Basrah

Abstract:

Samples from raw water and processing tanks (biological, chemical and final tanks) of general company of paper industries in Basrah (Paper Factory) were collected over a period of June to November 2001. Total plate -count, and total, faecal coliform counts showed an increasing numbers in the first three months of the study especially in July. The isolated bacteria from wastewater of final tank were identified and found that most of these were referred to Gram negative bacteria especially Escherichia coli, while only Bacillus sp. was referred to Gram positive bacteria. Correlation between antibiotic and mercuric chloride resistance was studied, the isolates were resistant to antibiotics showed resistance to mercuric chloride even at 10 mM.

Introduction:

Mercury is an extremely toxic metal in several forms and originates from industrial sources such as wastewater effluent and airborne particle deposition (1). Since mercury accumulates in body tissues, it can cause serious effects to the nervous system, kidney, or intestine at low doses over a long period of time. Mercury compounds become concentrated in the tissues of fish; therefore, fish taken from mercury polluted water should not be eaten(2).

Mercury possess a toxic effect on bacteria, this effect called "Oligodynamic action", in which high concentrations. of mercury cause coagulation to the cytoplasm, in this manner cell will be died (3), bacteria (Pseudomonas sp.) isolated from mucous material on the surface of fish and soil were able to methylate mercury under aerobic conditions. The purpose behind this process is to resist the higher concentrations of the toxic metal (4). On the other hand, the apparent increase of the occurrence of antibiotic resistance among bacteria from aquatic environment and especially from wastewater during the past years and its possible implications for public health (5) have in many countries lead to an intensified surveillance of bacterial resistance.

The work described in this study was performed to compare the mercuric chloride and antibiotic resistance among bacterial genera isolated from wastewater of Paper Factory in Basrah to test the hypothesis that heavy metal contamination may influence the occurrence of antibiotic resistance in the flora of an ecosystem contaminated by heavy metals.

Materials and Methods:

Sampling and culture conditions: The study extended over a period of six months from June-November 2001. Samples were taken monthly from raw water, biological, Chemical and final tanks of the Paper Factory.

Water samples were collected in sterile Nalgene polycarbonate flasks 250 ml. The samples were placed in ice box and transported to the laboratory within 3 h. From each sample 10 ml were taken and filtered through 0.45 µ. Millipore filter paper (WCN, Japan), cultured on plates containing the following media: Nutrient agar (Oxoid) to determine the total plate counts (TPCs) and MacConkey agar (Oxoid) to determine total coliforms (TCs) and faecal coliforms (FCs), the plates were incubated for 18hrs at 35°C for both TPCs and TCs, while they incubated for 48hrs at 44.5°c in water bath for FCs, the grown colonies were multiplied by 10 in order to record the results as cfu/100ml(6).

Identification of isolates: The isolated bacteria from

the final tank of the factory were identified according to (7), the isolates which follow each genus were counted monthly.

Preparation of inoculums: From each bacterial genus,

100 isolates were chosen randomly to complete the study, five colonies from each isolate were propagated in a vial containing 10 ml of nutrient broth, incubated at 35°C for 18 hrs. suspended in saline with a turbidity equivalent to that of 0.5McFarland standards, so the bacterial broth contain 1.5x10 cfu/ml(8).

Antibiotic susceptibility test: This test was performed

by the disk diffusion method (9). Ten antibiotics discs were used Ampicillin (AMP), 30µg; Nalidixic acid (NA) 30 µg; Penicillin G (PEN) 10 units; Erythromycin (E) 15 µg; Chloramphenicol (C) 30µg; Sulphamethoxazole (SUL) 500µg; Nitrofurantoin (FD) 300µg; Tetracycline (TE) 30µg; Rifampin (RA) 30µg; Streptomycin (S) 10µg.

The results were recorded according to (10), percentage of isolates fully resistant to the antibiotics were calculated.

Mercury resistance test: Mercury resistance was

performed according to (11). 1ml of each bacterial inoculum was spread on plates of nutrient agar supplemented with serial concentrations from 1 to 10 mM mercuric chloride which prepared according to law of molarity. after incubation for 18 hrs. at 35°C, the results were recorded as the percentage of isolates which gave a positive growth in each concentration.

Results and Discussion:

As shown in Table 1 there was a significant variation in the bacterial counts during the period of the study, there was increasing in bacterial numbers in the first three months especially in July, while in the second three months the bacterial numbers were decreased gradually especially in November. These findings are in agreement with (12) who attributed the high counts of bacteria in July to lower flows and high temperature that induced bacteria to consume the nutrients (mainly cellulose), so the bacterial numbers became high. In the second three months, the temperature began to below, the flows became higher, this led to decrease in bacterial counts. FCs were the lowest bacterial numbers and TCs ranged between FCs and TPCS.

Table 2 showed that Gram negative bacteria were the dominant among the bacterial genera isolated from wastewater of the final tank (88.77%). Seven genera were identified as Escherichia coli (31.85%), Proteus vulgaris (13.06%), Klebsiella sp. (10.95%). Achromobacter sp. (10.73%), Pseudomonas sp. (10.17%), Enterobacter cloacae (6.17%) and Flavobacterium sp. (5.84%), only Bacillus sp. (11.23%) was referred to gram positive group. These results are in agreement with (13), (14) who considered that the presence of pathogenic bacteria like Escherichia coli, Klebsiella sp., Proteus vulgaris, and Pseudomonas sp. in wastewater represents the greatest threat to public health; especially when the receiving water is used for domestic, recreational or agricultural purposes.

Table (1) Counts of bacteria (CFU/100 ml) isolated from different sites of paper factory in Basrah

Site			Raw water	Biological tank	Chemical tank	Final tank
Months (2001)	June	TPC	$7.3x10^9$	$6.7x10^8$	$4.2x10^5$	$3.8x10^5$
		TC	$4.8x10^8$	$3.9x10^8$	$2.1x10^6$	$3.7x10^7$
		FC	$3.9x10^2$	$3.7x10^7$	$2.2x10^5$	$4.3x10^5$
	July	TPC	UC	UC	UC	UC
		TC	$5.8X10^9$	$5.2X10^9$	$6.3X10^7$	$7.2x10^8$
		FC	$7.2X10^8$	$8.7X10^7$	$4.1X10^6$	$3.8x10^6$
	[illegible]	TPC	$8.2X10^9$	$7.9X10^9$	$5.8X10^8$	$4.8x10^8$

		TC	$5.7X10^{8}$	$6.9X10^{8}$	$2.7X10^{5}$	$3.2x10^{6}$
		FC	$3.9X10^{7}$	$2.8X10^{7}$	$1.3X10^{6}$	$2.7x10^{7}$
	Septemb.	TPC	$8.3X10^{6}$	$5.7X10^{5}$	$3.2X10^{5}$	$4.8x10^{6}$
		TC	$7.2X10^{4}$	$3.7X10^{4}$	$1.2X10^{4}$	$2.7x10^{4}$
		FC	$3.8X10^{3}$	$5.2X10^{3}$	$8.3X10^{2}$	$6.7x10^{3}$
	October	TPC	$2.9X10^{4}$	$7.8X10^{4}$	$7.5X10^{4}$	$3.0x10^{4}$
		TC	$2.9X10^{3}$	$1.7X10^{3}$	N.G	$3.0x10^{3}$
		FC	$8.0X10^{2}$	$5.0X10^{2}$	$2.0X10^{2}$	$1.0x10^{2}$
	Novemb.	TPC	$2.7X10^{4}$	$1.0X10^{4}$	$6.4X10^{2}$	$7.3x10^{3}$
		TC	$5.2X10^{3}$	$4.6X10^{3}$	N.G.	$3.5x10^{3}$
		FC	$7.2X10^{2}$	$3.0X10^{2}$	N.G.	$1.2x10^{2}$

TPC: Total plate count; TC: Total coliform; FC: Faecal coliform; N.G.: No Growth; UC: Uncountable

Table (2) Frequency of bacterial genera isolated from the final tank of Paper Factory in Basrah during 6 – months period

No.	Genus o species	No. of isolate	% of Total
1	*Escherichia coli*	573	31.85
2	*Achromobacter* sp.	193	10.73
3	*Flavobacterium* sp.	105	5.84
4	*Klebsiella* sp.	197	10.95
5	*Enterobacter cloacae*	111	6.17
6	*Protus vulgaris*	235	13.06
7	*Pseudomonas* sp.	183	10.17
8	*Bacillus* sp.	202	11.23

- **Total of isolates was 1799**

- Percentage of Gr genera was 88.77

Results of antibiotic susceptibility test for these bacterial species are summarized in Table 3 which showed a multi - drug resistance to antibiotics used in the study, three bacterial genera were fully resistant to all antibiotics used, Escherichia coli. Bacillus sp., and Pseudomonas sp., the other genera graduated in their resistance to the antibiotics, Proteus vulgaris was the most susceptible genus, these results are in agreement with (15). Studies of the epidemiology, genetics, and biochemistry of drug resistant bacteria indicate that the origin, selection, spread, and prevalence of these bacteria resulted from abuse of drugs, so there is a need for periodical bacteriological examination of wastewater and restriction in the use of antibiotics (16,17,18).

Table (3): Percentage of isolates fully resistant to the antibiotics

	Isolates	*E. coli*	*F.* sp.	*P. vulgaris*	*K.* sp.	*E.cloacae*	*A.* sp.	*P.* sp.	*B.* sp.
Antibiotics	AMP 30µg	100	73	83	97	98	82	100	100
	NA 30µg	100	90	87	58	87	51	93	100
	PEN 10 units	100	88	35	81	92	80	88	100
	E 15µg	100	75	43	87	91	95	96	100
	C 30µg	100	40	24	49	61	30	95	100
	SUL 500µg	100	N.D.	33	92	78	93	97	100
	FD 300µg	100	87	42	79	89	81	92	100
	TE 30µg	100	76	38	85	36	25	99	100
	RA 30µg	100	N.D.	87	93	N.D.	97	90	100
	S 10µg	100	23	47	ND	N.D.	N.D.	87	100

N.D. Not done; *E. coli*: *Escherichia coli*; *F.* sp.: *Flavobacterium* sp.; *P. vulgaris*: *Protus vulgaris*; *K.* sp.: *Klebsiella* sp.; *E. cloacae*: *Enterobacter cloacae*; *A.* sp.: *Achromobacter* sp.; *B.* sp.: *Bacillus* sp.; *P.* sp.: *Pseudomonas* sp.
AMP(Ampicillin); NA(Nalidixic acid); PEN(Penicillin); E(Erythromycin); C (Chloramphenicol); SUL (Sulphamethoxazole); FD (Nitrofurantion); TE (Tetracyclin); RA (Rifampin); S (Streptomycin)

The results of mercuric chloride resistance test are shown in Table 4, species Proteus vulgaris was the least resistant to this substrate, while species Escherichia coli, Pseudomonas sp., and Bacillus sp. were resistant to mercuric chloride even at 10mM From these results, one can observe the correlation between high levels of resistance to mercuric chloride and antibiotics, this is in agreement with(19, 20).Heavy metal resistance in a number of different bacterial species have been shown to be plasmid mediated (21) and in some cases is present together with antibiotic resistance(19). In instances where these genes are grouped on the same plasmid, it is reasonable to assume that either heavy metals or antibiotics could serve as selection pressure for populations of bacteria hosting these plasmids (20) However, the nature of the factors contributing to these metal - resistant microorganisms has not yet been explained by epidemiological and genetic investigation. These metal resistant isolates -do not appear to originate by chance, it presumed that one of the factors selecting for metal resistant microorganisms may be environmental - contamination by these metals (16,22). This contamination results from the increased usage of metals in industry, thus, and according to our results We suggest restriction use of mercury in paper industry.

Table (4)Percentage of isolates fully resistant to Mercuric Chloride

Isolates		*E. coli*	*F.* sp.	*P. vulgaris*	*K.* sp.	*E.cloacae*	*A.* sp.	*P.*sp.	*B.* sp.
Mercuric Chloride (mM)	10	100	N.G.	N.G.	N.G.	N.G.	N.G.	72	100
	9	100	18	N.G.	N.G.	N.G.	N.G.	79	100
	8	100	36	N.G.	N.G.	N.G.	N.G.	88	100
	7	100	38	N.G.	N.G.	N.G.	N.G.	94	100
	6	100	49	N.G.	N.G.	N.G.	N.G.	98	100
	5	100	51	N.G.	N.G.	N.G.	11	100	100
	4	100	100	11	35	20	55	100	100
	3	100	100	38	43	61	73	100	100
	2	100	100	43	65	73	100	100	100
	1	100	100	57	68	85	100	100	100

N.G. No. Growth; *E. coli*: *Escherichia coli*; ***F.* sp.:** ***Flavobacterium* sp.; *P. vulgaris*: *Protus vulgaris*;*K.* sp.:*Klebsiella* sp.;**
***E. cloacae*: *Enterobacter cloacae*;*A.* sp.:** ***Achromobacter* sp.; *B.* sp.: *Bacillus* sp.; *P.* sp.: *Pseudomonas* sp.**

References:

1-World Health Organization (1997). Guidelines for drinking water quality. Health criteria and other supporting information., vol. 2, 2nd edition. Geneva, reprinted in Amman, Jordan. PP: 285-293.

2-Teitzel, G. M. and Parsek, M. R. (2003). Heavy metal resistance of biofilm and planktonic Pseudomonas aeruginosa. Appl. Environ. Microbiol. 69(4): 2313 2320.

3-Ibrahim, A. M. (1988). Heavy metal inhibition of resting nitrifying bacteria. In: 34 Industrial waste conference., J. M. Bell. (ed.), Lewis Publishers, Inc. chisea. PP: 309 -311.

4-Wood, J. M. and Wang, H. K. (1983). Microbial resistance to heavy metals. Environ. Sci. Technol. 17: 82-90.

5-Jazrawi, S. F.; Al - Doori, Z. A. and Haddad, T. A. (1988). Antibiotic resistant coliform and faecal coliform bacteria in drinking water. Water air soil poliut. 34, (3-4): 377-3S2.

6-Partram, J.; and Welter, D. (1997). Principles for planning and implementation of microbiological analyses. In: Global Environmental Monitoring System (GEMS). Ch 5, PP: 1-9.

7-Holt, J. G.; Krieg. N. R.; Sneath, P. H. A.; Staley, J. T. and Williams, S. T. (eds.) (1994). Bergey's manual of determinative bacteriology. 9th ed., 787 PP.

8-Marques, M. B.; Brookings, E. S.; Moser, S. A.; Snoke, P. B. and Walts, K. B. (1997). Comparative in vitro antimicrobial susceptibilities of nosocomial isolates of Acinetobacter baumannii and synergistic activities of nine antimicrobial combinations. Antimicrob. Agent. Chemother.41 (5): 881-885.

9-Baron, E. J. and Finegold, S. M. (1990). Diagnostic microbiology, 7th ed. The C. V. Mosby Company. Toronto, pp: 171-185.

10-National Committee for Clinical Laboratory Standards. (1994). Performance standards for antimicrobial susceptibility testing: fifth informational supplement. NCCLS document M 100-S5, vol. 14. National Committee for Clinical Laboratory Standards. Villanova, Pa.

11-Dhakephalkar, P. K. and Chopade, B.A. (1994). High levels of multiple metal resistance and its correlation to antibiotic resistance in environmental isolates of Acinetobacter. Biometals. 7(1): 67-74.

12-Murray, D. and Coots, R. (2003). Colvill river watershed bacteria, total maximum daily load.

Submittal report Amended. Washington State Department of Ecology. Publication No. 03-10-030, Olympia, USA.

13-Feresu, S. B. and VanSickle, J. (1990). Coliforms as a measure of sewage contamination of the River Zambezi. J. Appl. Microbiol. 68: 397-403.

14-John, D. E.; Rose, J. B. and Kamarainen, A.; (2004). Survival of fecal indicator bacteria, bacteriophage and protozoa in Florida s surface and ground water: potential implications for aquifer storage and recovery. Final report of the fate of microorganisms in aquifers. Study funded by the south west Florida water management. USA. 240pp.

15-Goni-Urriza, M.; Capdepuy, M.; Arpin, J.; Raymond, N.; Caumette, P. and Quentin, C. (2000). Impact of an urban effluent on antibiotic resistance of riverine Enterobacterieceae and Aeromonas spp. Appl. Approx.

Microbiol. 66(1): 125-132.

16-Nakahara, H.; Ishikawa, T.; Sarai, Y.; Kondo, 1.; Kozukue, H. and Silver, S. (1977). Linkage of mercury, cadmium, and arsenate and drug resistance in clinical isolates of Pseudomonas aeruginosa. Appl. Environ. Microbiol. 33(4): 975-916.

17-Morozzi, J.; Sportolari, R. and Caldini, G. (1988). The effect of aerobic and anaerobic wastewater treatment on faecal coliform and antibiotic resistant faecal coliforms. Zentralbl. Batteriol. Microbiol. Hyg. Abt. B. 185 (4-5): 340-349.

18-HuYs, G.; D'Haene, k.; VanEldere, J.; VanHoly, A. and Swings, J.; (2001). Incidence, distribution, and spread of tetracycline resistant determinants and integron associated antibiotic resistant genes among motile aeromonads from a fish farming environment. Appl. Environ. Microbiol.

67 (12): 5675 5682.

19-HuYs, G.; D, Haene, k.; VanEldere, J.; VanHoly, A. and Swings, J.; (2005). Molecular diversity and chacterization of tetracycline resistant Staphylococcus aureus Isolated from a poultry processing plant. Appl.

Environ. Microbiol. 7(1): 574-579.

20-Nemergut, D. R.; Martin, A. P. and Schmidt, S. K.;

(2004). Integron diversity in heavy-metal contaminated mine tailing and interferences about integron evolution.

Appl. Environ. Microbiol. 70 (2): 1160-1168.

21- Hermansoon, M.; Jones, G. W. and Kjelleberg. S.; (1987). Frequency of antibiotic and heavy metal resistance, pigmental, and plasmids in bacteria of marine air water interface. Appl. Environ. Microbiol. 53 (10)

: 2338-2342.

22- Timoney, J. F.; Port, J.; Giles, J. and Spanier, J.; (1978).

Heavy metal and antibiotic resistance in the bacterial flora of sediments of New York bight. Appl. Environ.

Microbiol. 36 (3): 465-472.